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Etifoxine analgesia in experimental monoarthritis: A combined action that protects spinal inhibition and limits central inflammatory processes

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ABSTRACT

Inflammatory and degenerative diseases of the joint are major causes of chronic pain. Long-lasting pain symptoms are thought to result from a central sensitization of nociceptive circuits. These processes include activation of microglia and spinal disinhibition. Using a monoarthritic rat model of pain, we tried to potentiate neural inhibition by using etifoxine (EFX), a nonbenzodiazepine anxiolytic that acts as an allosteric-positive modulator of gamma-aminobutyric acid type A (GABAA) receptor function. Interestingly, EFX also can bind to the mitochondrial translocator protein (TSPO) complex and stimulate the synthesis of 3α-reduced neurosteroids, the most potent positive allosteric modulator of GABAA receptor function. Here we show that a curative and a preventive treatment with 50 mg/kg of EFX efficiently reduced neuropathic pain symptoms. In the spinal cord, EFX analgesia was accompanied by a reduction in microglial activation and in the levels of proinflammatory mediators. Using electrophysiological tools, we found that EFX treatment not only amplified spinal GABAergic inhibition, but also prevented prostaglandin E2–induced glycinergic disinhibition and restored a "normal" spinal pain processing. Because EFX is already distributed in several countries under the trade name of Stresam for its anxiolytic actions in humans, new clinical trials are now required to further extend its therapeutic indications as pain killer.

1. Introduction

Inflammatory and degenerative diseases of joints are major causes of chronic pain [6]. To better understand the pathophysiological mechanisms associated with joint pain and to design new therapeutic strategies, chronic inflammatory pain resulting from a unilateral intra-articular knee injection of complete Freund's adjuvant (CFA) has been well characterized [8]. CFA induces several plastic changes in the nociceptive pathways, including sensitization of joint nociceptors [18,42] and of central neurons [31,43]. These processes are thought to account for the expression of spontaneous pain behaviors and pain symptoms [41].

Proinflammatory mediators, overproduced at the injured site [37] and in the spinal cord, contribute in large part to the early phase and maintenance of sensitization processes [38,46,50]. For example, neutralization of tumor necrosis factor α (TNFα), directly at the joint or in the spinal cord of monoarthritic rats, produces significant antinociception [4,5]. Increased prostaglandin E2 (PGE2) synthesis and release has been demonstrated clearly in the spinal cord of rats suffering from monoarthritic pain [51] as a result of overexpression of the inducible PGE2-synthesizing enzyme cyclooxygenase type 2 [16]. Agreeing with these findings is research showing that intrathecal application of PGE2 produces significant excitation of spinal cord neurons in naive rats [49], and increases pain symptoms when injected in vivo [29,30,48]. Further studies have revealed that PGE2-induced central sensitization and pain symptoms result from a loss of glycine receptor–mediated synaptic inhibition [1], achieved by the activation of prostanoid EP2 receptors [35] and by hyperphosphorylation of α3 subunit–containing glycine receptors (GlyRs), expressed by spinal neurons in the most superficial dorsal horn layers [21]. If proinflammatory cytokines can be released by activated microglia and astrocytes within the nociceptive system, they also can secrete trophic factors, such as brain-derived neurotrophic factor (BDNF). Microglial BDNF recently was found to be involved in the downregulation of KCC2 potassium chloride exporters in the spinal cord of neuropathic rats

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[12]. KCC2 downregulation also has been found to deeply alter chloride gradients in nociceptive-specific spinal neurons and to reduce inhibitory chloride currents mediated by gamma-aminobutyric acid type A (GABAA) receptors (GABAARs) and GlyRs [13].

In summary, preventing PGE2-induced glycinergic disinhibition in the spinal cord and BDNF-mediated downregulation of KCC2 expression is an interesting option to limit spinal nociceptive processing and pain symptoms. Another strategy could consist of potentiating the remaining GABAAR-dependent inhibitory controls using positive allosteric modulators such as etifoxine (EFX), a non-benzodiazepine anxiolytic [28,32,44] binding preferentially to β2 subunits [19]. EFX also stimulates the synthesis of neuroactive steroids [39] such as the allopregnanolone, a 3α-reduced neurosteroid (3αNS) with potent analgesic properties [9]. In a chemotherapy-induced neuropathic pain model, EFX treatment fully alleviated pain symptoms [2] and these pain symptoms never reappeared after the end of the treatment. This observation is unlikely to be solely explained by direct (allosteric) and indirect action (production of endogenous allosteric modulator: 3αNS) on GABAARs but rather due to long-term changes in gene expression and possible local chloride homeostasis.

We tested this hypothesis on inflammatory-driven pain symptoms in rats using the well-characterized CFA-induced monoarthritic model of pain. We analyzed the possible effects of EFX on the central inflammatory response and its consequences on spinal inhibition.

2. Methods

2.1. Animals

In this study, male Sprague-Dawley rats 250 to 350 g (Janvier, Le Genest St. Isle, France) were housed in groups of 3 under standard conditions (room temperature: 22°C; 12/12-hour light–dark cycle) with ad libitum access to food and water. All experiments were conducted in conformity with the recommendations of the European Committee Council Direction of September 22, 2010 (2010/63/EU). Procedures were positively evaluated by the regional ethical committee, and experiments were conducted with an official authorization for animal experimentation from the French Department of Agriculture (License 67-116 to P.P.).

2.2. Behavioral testing

All animals were habituated to the room and to the tests at least 1 week before starting the experiments. Mechanical nociceptive thresholds were measured using a calibrated forceps (Bioseb, Vitrolles, France) as previously described [2]. Briefly, the habituated rat was loosely restrained with a towel masking the eyes in order to limit stress by environmental stimulation. The tips of the forceps were placed at each side of the paw, and a gradually increasing force was applied. The pressure, in grams, producing withdrawal of the paw or in some rare cases the vocalization of the animal, corresponded to the nociceptive threshold value. This manipulation was performed 3 times for each hindpaw, and the values were averaged. All tests were performed between 10:00 am and 4:00 pm prior to any injection.

2.3. Drugs and treatments

Inflammation was induced by unilateral knee injection of 50 μL CFA (Sigma, St. Louis, MO) following the procedure already published on the tibiotarsal model [8]. The control animals received an equivalent volume of mineral oil, the vehicle of CFA (Sigma). Etifoxine (EFX: Biocodex, Gentilly, France) was prepared in saline (NaCl .9% in distilled water) containing 1% Tween 80 (v/v; Sigma) and injected daily (50 mg/kg intraperitoneally in a final volume of 10 mL/kg). In previous studies, this concentration has been shown to fully alleviate pain symptoms when neuropathic animals received EFX as a prophylactic and/or curative treatment [2,3]. This vehicle solution was used (without EFX) for the control group. To study the effect of etifoxine on pain symptoms, 5 consecutive daily injections were given to rats, 3 days after the CFA injection. Prophylactic EFX treatment started 1 week before the CFA injection and lasted for 3 weeks.

2.4. Quantitative polymerase chain reaction (PCR)

Lumbar spinal cord was collected at day 9, after the 5 injections of EFX (or vehicle) and directly stored at −80°C. Total RNA was extracted according to a protocol consisting of 2 independent total RNA extractions separated by a DNAseI treatment (DNA-free kit, Ambion, Life technologies, Saint Aubin, France) as previously described in detail [24]. RNA quality and concentration were assessed by spectrophotometry and automated electrophoresis on microfluidic chips (Agilent 2100 Bioanalyzer system, Agilent technologies, Les Ulis, France). Total RNA (800 ng/sample) was subjected to reverse transcription using the Iscript kit according to the manufacturer instructions (Bio-Rad, Marnes-la-coquette, France). PCR was set up in 96-well plates using diluted cDNA samples, highly selective primer sets (see sequences in Supplementary material), and SyberGreen-containing PCR reagents (Bio-Rad) accurately dispensed using a robotic workstation (Freedom EVO100 from Tecan, Lyon, France). Gene amplification and expression analyses were performed on a MyIQ real-time PCR machine (Bio-Rad) using a 3-step procedure (15 seconds at 96°C; 10 seconds at 62°C; 15 seconds at 72°C) followed by a melting curve study to ensure specificity of the amplification process. Standardization was made possible using standard curves made from control RNA samples and hypoxanthine phosphoribosyltransferase 1 as housekeeping gene. The differences between samples were calculated on the basis of the specific ratios (gene of interest/housekeeping gene).

2.5. Immunohistochemistry for OX-42

On day 5 of the EFX treatment, rats were perfused intracardially with 150 mL of phosphate buffer (0.1 M, pH 7.4) followed by 500 mL of a solution containing 4% paraformaldehyde .6%, picric acid in phosphate buffer. After laminectomy, the lumbar spinal cord (L2–L5) segments were collected, immersed overnight in the same fixative, and washed the next day in phosphate-buffered saline (PBS). When collected, the lumbar spinal cord was notched on the ventral right side to allow both sides to be distinguished (ipsilateral vs contralateral to the injected knee). Transverse sections of 40 μm were prepared using a tissue slicer (Leica VT1000S, Wetzlar, Germany). The sections were rinsed 3 times during 10 minutes in PBS and subsequently were incubated for 1 hour in a blocking solution composed of 5% donkey serum in PBS and .5% Triton X-100. The sections were then incubated overnight with the primary antibody OX-42 (1:400, Serotec, ref. MCA275R, Raleigh, USA) diluted in PBS containing 1% normal serum and .5% Triton X-100. After a wash with PBS, the sections were incubated for 90 minutes with the secondary biotinylated antibody (1:2000, Santa Cruz Biotechnology Inc., Dallas, USA) and treated with a peroxidase-conjugated avidin-biotin complex (Vectastain Elite ABC Kit, Vector Laboratories) for 1 hour at room temperature. After rinsing with PBS (3 × 10 minutes), ABC reaction was revealed by incubation with 3,3′-diaminobenzidine tetrahydrochloride, .03% H_2O_2 in .05 mL Tris buffer (pH 7.6). The reaction was stopped with distilled water, and the sections were rinsed for 10 minutes with Tris-HCl buffer (pH 7.6) and with PBS (2 × 10 minutes) before being mounted on gela-